Chapter 1

Introduction
1.1 The world of Neurospora

The naïve appearance of the red mould Neurospora, might deceive us into thinking it to have a very routine biology. Yet to the scientific world, it has turned out to be one of the most exciting model systems, willing to provide answers to any questioners seeking them. Commonly called as the bread mold, Neurospora was originally discovered as a contaminant in bakeries. It can grow naturally on tree barks, burnt sugarcane stubble, bagasse heaps and on roasted corn-cobs (Fig 1.1). It presents a tractable system for biochemical and genetic analysis. Neurospora crassa has been pivotal in the origination of important hypotheses made based on research done using it as a model system. For example the ‘one gene one enzyme’ hypothesis came from the pioneering biochemical work done using N. crassa (Beadle and Tatum, 1941; 1945).

Not only is the fungus harmless but in some parts of the world it is gastronomically prized. The edible variety of Neurospora is N. sitophila and is colloquially known as ‘Ontjum’. Apart from N. crassa, there are other species, which are broadly classified as homothallic, pseudohomothallic and heterothallic. N. africana, N. dodgei and N. lineolata are the true homothallic species. The heterothallic species are N. crassa, N. intermedia, N. sitophila and N. discreta. N. tetrasperma (Dodge, 1927; Raju and Perkins, 1994; Bistis, 1996) is the pseudohomothallic species. The Neurospora strains isolated from different regions of the world (referred to as wild-isolated strains) have been studied and classified at the Fungal Genetics Stock Center (FGSC) where stringent procedures for classification of a wild isolated culture are followed. The conidial cultures from the site are purified several times before further analysis. Using tester strains of various type species individual strains have been assigned to one of the species N. tetrasperma, N. crassa, N. intermedia, N. discreta and N. sitophila. The first criterion to establish the species of a strain is whether they produce a minimum of 90% black spores in crosses (Turner and Fairfield, 2001). Wild-isolated strains can be useful and informative in several ways. Based on the relatedness of species, many aspects of mitochondria and horizontal transfer of mitochondrial plasmids can be studied using the wild isolates (Griffiths and Yang, 1995; Griffiths, 1995). They might also reflect upon the way alleles for different loci were selected. It was among the wild-isolated cultures of N. intermedia that spore killer elements were discovered (Campbell and Turner, 1987).
Figure 1.1: Neurospora growing in the wild. A. Neurospora blooms on the bark of a tree. B. Neurospora growth on sugar cane. C. Neurospora growing in mud near a distillery waste dumping site. Images A and B taken from FGSC website, Dr. D. Jacobson's collection and image C from contributions made to the FGSC website by Dr. Maruthi Mohan (Rashmi et al, 2003)
Based on DNA:DNA homology, the pseudo-homothallic species are closer to the heterothallic species than to the true homothallic species (Dutta et al., 1976).

1.2 *Neurospora crassa* as a model system

*N. crassa* has been preferred as a model system by most researchers because of the less complex genetics and the ease with which it can be handled. Being a haploid organism with a relatively short life cycle, genetic analysis in *N. crassa* is easy and there are a large number of mutants available.

1.2.1 Life cycle of *Neurospora crassa*

*Vegetative stage*

*N. crassa* is a heterothallic fungus and its asexual and sexual life cycles are clearly demarcated (Fig. 1.2). Vegetative propagation in *N. crassa* occurs by means of the substrate mycelia, which have dividing cells at the tips. They branch out and spread over the substratum. They also give rise to arial hyphae, which bear the vegetative spores called conidiospores (or conidia). These spores have the ability to disperse and germinate when they come in contact with a suitable substrate. The conidia are of two types, macroconidia, which are multinucleate and microconidia, which are uninucleate. Most part of the life cycle of *N. crassa* is spent as a haploid organism. It undergoes sexual reproduction when starved for nitrogen.

*Sexual cycle*

*N. crassa* is a Pyrenomycete (Perkins and Barry, 1977) having perithecia or flask shaped sexual fruiting bodies. Presence of two different mating types in *N. crassa* makes it a bipolar mating type system.

*Mating types in N. crassa*

The two mating types, designated *mat A* and *mat a*, have unrelated sequences, and hence they have been termed ‘idiomorphs’ (Newmeyer et al., 1973; Metzenberg and Glass, 1990; Staben and Yanofsky, 1990). The mating type alleles are located on LGIL.
Figure 1.2: Schematic representation of the life cycle of *Neurospora crassa*. The specific stages of the life cycle in which the three different genome defense mechanisms identified in Neurospora are active have been shown. Adapted from Shiu et al., 2001.
The mat $A$ idiomorph encodes three genes, mat $A$-1, mat $A$-2 and mat $A$-3 whereas the mat $a$ idiomorph codes for only one gene, mat $a$-1. Mating specificity is imparted by the mat $a$-1 and mat $A$-1 genes (Glass et al., 1988; Glass et al. 1990; Glass and Lee, 1992; Ferreira et al., 1996; Ferreira et al., 1998). The maintenance of nuclear identity and recognition of 'self' from 'non-self' are among the functions of the mating type gene products (Metzenberg, 1990; Nelson and Metzenberg, 1992; Nelson, 1996).

The mating type alleles also control vegetative incompatibility. (A+a) cultures are inhibited in growth and this vegetative incompatibility of the mating type heterokaryon (described as the vegetative existence of a cell that has the genetic composition of two different nuclei) can be suppressed by the $tol$ mutation (Jacobson, 1992; Smith et al., 1996). Carboxyl terminal of the mat $a$-1 polypeptide controls both the mating type specificity and the vegetative incompatibility, which is a biochemically distinct mechanism (Philley and Staben, 1994). Other than the mating type alleles there are several other genes categorized as the het genes that determine the compatibility of a heterokaryon. Stable heterokaryons can form only when the participating nuclei have genetic identity at all the ten het loci thus far identified in $N. crassa$. Heterozygosity for any one of the het loci in a heterokaryon can cause killing of the cells that have unlike nuclei (Garnjobst and Wilson, 1956; Mylyk, 1975; Saenz et al., 2001). Heterokaryons can be very helpful in rescuing mutant phenotypes that are otherwise difficult to maintain.

Fertilization and ascospore development

In the sexual cycle, either of the mating types can participate as the male or the female parent and conidia serve as the male parent (Alexopoulos and Mims, 1979). Fertilization results in the transformation of a protoperithecium into a fruiting body called perithecium (Fig. 1.3 A) and is mediated by a specialized hypha, trichogyne (Dodge, 1935; Backus, 1939; Bistis, 1983; Kim and Borkovich, 2006). Pheromones serve in the attraction of the trichogyne to the conidia of the opposite mating type. The two haploid nuclei of the opposite mating type remain in close association and divide by several conjugate divisions in a specialized structure called crozier before finally fusing. In the pseudohomothallic species, each ascospore has two mating type nuclei therefore they
Figure 1.3 A: Structure of a mature perithecium of *N. crassa*. A mature perithecium of Neurospora has the ascogenous tissue. Fertilization occurs within this tissue which later develops and gives rise to a rosette of sac-like structures called asci which have ascospores within them. The opening on the top, ostiole helps in spore dispersal. Image from Johnson T E (1978). Genetics 88: 27-47.
have the ability to self-mate. The zygote is the only diploid cell and is short lived. It immediately undergoes meiosis and a post-meiotic mitosis to give rise to eight nuclei that are aligned in a linear manner in a sac-like tissue called the ascus (Fig 1.3 B). The true homothallic and heterothallic species have eight-spored asci. Instead of the usual eight-spored asci, *N. tetrasperma* has four-spored asci. Ascospores in *N. tetrasperma* are initially binucleate whereas those in the other species are initially uninucleate. In *N. tetrasperma* there is an overlap of the spindles during the second meiotic division. This brings two nuclei of opposite mating types in close proximity and they are portioned into a single ascospore (Fig. 1.3 C; Pincheira and Srb, 1969; Raju and Perkins, 1994). In *N. crassa* homozygosity for a recessive mutation can be generated only by having the mutation in both parents participating in a cross. Second division segregation of some markers in *N. tetrasperma* may create homozygosity for the markers, which can be maintained in the ascospore. This difference of ascospore development in *N. tetrasperma* (Novak and Srb, 1971) can be taken advantage of to identify recessive mutations affecting processes specific to the diplophase (Bhat *et al.*, 2004). After delimitation and further maturation, the ascospores are forcibly discharged from the asci to long distances. Many genetic studies make use of the fact that the ascospores in *N. crassa* asci are linearly arranged in a manner that represents the meiotic segregation. They can be maintained in the dormant state practically forever.

### 1.2.2 Genome constitution of *Neurospora crassa*

The other feature that has opened up newer opportunities making *N. crassa* a better model system is the availability of the genome sequence (Perspective article of Davis and Perkins, 2002). *N. crassa* has seven linkage groups (LG) and of these LG1 is the largest (Lindegren, 1933; 1942; McClintock, 1945; Houlahan *et al.*, 1949; Barratt and Garnjobst, 1949; Barratt *et al.*, 1954). The genome of *N. crassa* is about 40 Mb with ~10,000 protein-coding genes (Galagan *et al.*, 2003). The number of genes is twice that in *Schizosaccharomyces pombe* and almost equivalent to that in *Drosophila melanogaster*. Nearly 41% of the proteins coded by these genes are novel and do not have any significant match in the public databases. There are far fewer genes in multigene families and only about eight genes that possess a homologue with more than
Figure 1.3 C: (i) Meiosis and ascospore delimitation and development in the pseudohomothallic species, *N. tetrasperma*. Note that the specific arrangement of the spindle assembly causes the segregation of two nuclei in a single spore. Therefore the asci are four spored. (ii) A rosette of *N. tetrasperma* asci. Images taken from the FGSC website, Dr. N. Raju's collection.
80% homology. The genome sequence revealed presence of the red light sensing genes, which were unexpected considering the photobiology of Neurospora which was never known to involve any red light sensing genes (Galagan et al., 2003).

1.3 Genome stability and genome defense

Among the commonly known causes of genome instability in most organisms is the presence of active transposable elements. Transposition of such elements leads to their accumulation in multiple copies. Homologous recombination between these DNA sequences can bring about chromosomal rearrangements, which can affect the genetic fitness of an organism. They may also bring about silencing of the neighboring sequences and thus retard the proper functioning of the cell machinery and disrupt the genome stability. Although sexual reproduction has evolved as a means to provide variability and prevent rearrangements (reviewed by Zolan, 1995), it does not provide defense against such selfish DNA elements. Given the fact that genomes are prone to destabilization owing to external and internal factors, organisms have evolved mechanisms that can provide genome defense. Prokaryotes have de novo methylation (restriction modification) as a way of preventing proliferation of foreign genetic material (Tock and Dryden, 2005). Similarly, many eukaryotes have evolved novel mechanisms for genome defense. In Tetrahymena, chromosomes undergo rearrangements that are guided by an RNAi-dependent pathway and this nuclear reorganization serves as a good way of genome defense (Yao et al., 2003). DNA methylation as a method of genome defense is a well-known phenomenon and has been reported in many organisms. RNAi-mediated heterochromatinization of unpaired DNA is a means of genome defense in Caenorhabditis elegans, (Bean et al., 2004).

Genome defense in N. crassa

Most strikingly, the N. crassa genome is almost completely devoid of active transposable elements and there are hardly any repetitive elements. Like any other organism, the genome of Neurospora shows evidence for past invasion by transposable elements. Some wild-isolated strains of Neurospora were shown to harbor defective gypsy like elements dab-I (dead and buried). Other transposable elements like Punt and
the Tc1/Mariner super family element, *Guest* have been ancient invaders of the Neurospora genome (Bibbins *et al.*, 1998; Margolin *et al.*, 1998; Yeadon and Catcheside, 1995; Rammussen *et al.*, 2004). *Tad* is a LINE-like transposon that is found as inactivated copies in many Neurospora species. In the active form, these elements propagate by retrotranscription of RNA intermediates. They are not infective but active copies can be transferred to naïve strains by forcing heterokaryons or by transforming with the *Tad* elements (Kinsey, 1993a). They are capable of transnuclear retrotransposition, indicating the presence of a cytoplasmic phase in the retrotransposition events (Kinsey, 1993b). Introduction of a foreign transposable element, *Restless* in *N. crassa* genome by transformation resulted in methylation of the sequence (Kempken and Kuck 1996). The *Punt* transposable element DNA sequence also showed evidence of mutations suggesting that any transposable elements that entered the genome of Neurospora have been inactivated. Not only transposable elements but also any sequence that was present in multiple copies showed evidence of silencing. These findings indicated the existence of gene-silencing mechanisms that prevent proliferation of transposable elements and other repetitive sequences (Kempken and Kuck 1998). Neurospora seems to be a treasure trove of genome defense processes and three different gene-silencing processes have been identified thus far.

Gene silencing can be transcriptional (TGS), which prevents RNA synthesis or post-transcriptional (PTGS), which is an RNA-degradation process that blocks translation. PTGS in plants and RNAi in animals like *C. elegans* provides defense against retroviruses (Nakayashikiet al., 2005; Nakayashiki, 2005; Voinnet, 2001; Wilkins *et al.*, 2005). PTGS and RNAi both require dsRNA, putative RNA-dependent RNA polymerases (RdRP), RNA helicases and proteins containing PAZ and Piwi domains for silencing (Vaucheret H, 2001; Vaucheret *et al.*, 2001). The first reports of de novo gene silencing described decreased levels of pigment in petunia flowers when multiple copies of a gene imparting purple color to the flower were introduced (Van der Krol *et al.*, 1990). RNAi and chromatin based silencing has also been identified in *C. elegans* (Jones and Schedl 1995; Gaudet *et al.* 1996; Dernburg *et al.*, 1998, 2000; Ketting and Plasterk 2000, Robert *et al.*, 2005). The term RNA interference was introduced by Fire and Mello (Fire *et al.*, 1998) when they showed that double stranded RNA was able to direct the
degradation of messenger RNA (mRNA) with sequence complementarity to one or other strand. An enzyme called dicer (Bernstein et al., 2001) cleaves the dsRNA produced by the transgenes or the retro elements into 21 – 23 mer RNA duplexes called siRNA (Zamore et al., 2000). The siRNA are used by the RNA induced silencing complex (RISC) to identify complementary sequences on mRNA and destroy them. It has also now become clear that heterochromatic silencing in fungi involves the processing of transcripts of repeat sequences into siRNAs (Lippman & Martienssen, 2004).

1.4 Quelling

Observations in Neurospora revealed that the carotenoid biosynthesis gene al-1 expression was silenced when multiple transgene copies for the gene were present (Romano and Macino, 1992). In Neurospora, multiple copies of transgenes or transposable elements that might be present in the genome are silenced in an siRNA dependent way by a mechanism called quelling (Romano and Macino, 1992; Cogoni et al., 1994; Cogoni et al., 1996). It is active during the vegetative stage (Cogoni and Macino, 1997). The genes required for this mechanism are qde1, qde2 and qde-3 (Cogoni and Macino, 1999a; Cogoni and Macino, 1999b and Catalanotto et al., 2000). They code for an RdRP, an Argonaut protein and a REC-Q helicase respectively (Fig.1.4). Although a lot of study has been done on quelling, its role in genome defense is not clearly established in Neurospora.

Barbara McClintock observed that silent mobile genetic elements could be activated and inherited in the active state. This inheritance could be transient or permanent because sometimes silencing and activation cycles could last for generations (McClintock 1965). The exact mechanisms that underlie the altered state of the transposon activity remain obscure although DNA methylation has been implicated in maize (Chandler and Walbot 1986; Banks et al., 1988; Martienssen and Baron 1994, Lippman et al., 2003). Likewise, methylation status can also cause altered allele influence as in the case of the inactivated and methylated epialleles of PAI2 gene of Arabidopsis in the presence of a closely related gene, PAI1 (Melquist et al., 1999; Luff et al., 1999). In C. elegans, transposons may generate dsRNA and act as major targets for
Quelling
(Vegetative phase)

repeated DNA

aberrant RNA

Qde-1

dsignal amplification?
mobile signals?

degradation

Qde-2

dsiRNAs

cognate mRNA

Dcl-2

dsRNA

Figure 1.4: Proposed model for Quelling in *N. crassa*. During the vegetative phases of the *N. crassa* life cycle, repeated sequences in the genome can induce quelling. In this pathway, dsRNA produced by Qde-1 RNA-dependent RNA polymerase is diced into siRNAs mainly by the action of Dcl-2. The siRNAs guide degradation of cognate mRNA after their incorporation into RNA-induced silencing complex (RISC) where Qde-2 is one of the components. Figure taken from Nakayashiki *et al.*, 579, (2005) 5950-5957.
RNAi or they may be targeted for methylation using RNA as guide (Ramsahoye et al., 2000; Grishok, 2005).

Repeat-induced point mutation (RIP) in Neurospora and methylation induced premeiotically (MIP) in Ascobolus immersus (Barry et al., 1993; Rossignol and Faugeron 1994) are some transcriptional gene-silencing mechanisms. The methyl transferases masc-1 and masc-2 in Ascobolus and dim-2 and dim-5 in Neurospora (Malagnac et al., 1997; 1999; Kauzminova and Selker, 2001; Tamaru and Selker, 2001) are responsible for methylation in the two organisms. RIP in Neurospora is a robust genome defense process that causes silencing of genes by introducing mutations and not by methylation alone.

1.5 RIP (repeat-induced point mutation)

RIP is a mutational process, which occurs during the haploid dikaryotic stage of the sexual cycle and scans the genome for the presence of transposons and other duplicated DNA sequences. Any such sequences are altered irreversibly with multiple G:C to A:T changes (Fig. 1.5; Selker et al., 1987a and 1987b; Selker and Garrett, 1988; Cambareri et al., 1989; Selker, 1990). Many of the remaining cytosine residues are methylated, which severely affects transcription due to stalling of the RNA polymerase II (Selker et al., 1987; Cambareri et al., 1989; Selker et al., 1993; Singer et al., 1995a; Rountree and Selker, 1997). Frequency of RIP can vary with the age of the cross. Late ascospores generally show higher RIP frequency (Singer et al., 1995b). This is possibly because they are produced from many premeiotic mitoses and have therefore undergone repeated rounds of RIP.

The preferred sites for RIP are cytosine residues immediately 5' to adenine (CpA) residues, the other sites in order of preference are CpG, CpT and CpC (Cambareri et al., 1989; Grayburn and Selker, 1989; Selker, 1990). RIPed sequences have 80 % probability of acquiring an in-frame stop codon in a single round of RIP (Selker, 1995; Galagan et al., 2003). RIP can therefore be used to generate null alleles. RIPed sequences can undergo further mutations when put through another cross until the duplicated sequence is diverged by 30 % (Cambareri et al., 1991).
Repeat-induced point mutation (RIP)

\[
\begin{align*}
\text{CATGTACAGCA} \\
\text{GTACATGTTCGT}
\end{align*}
\]

\[
\begin{align*}
\text{CATGTACAGCA} \\
\text{GTACATGTTCGT}
\end{align*}
\]

\[
\begin{align*}
\text{CATGTACAGCA} \\
\text{GTACATGTTCGT}
\end{align*}
\]

\[
\begin{align*}
\text{TATGTATAGTA} \\
\text{ATACATACAT}
\end{align*}
\]

\[
\begin{align*}
\text{TATATAAGCA} \\
\text{ATATATTCGT}
\end{align*}
\]

\[
\begin{align*}
\text{TATGTATAGTA} \\
\text{ATACATATCAT} \\
\text{CH}_3 \quad \text{CH}_3
\end{align*}
\]

\[
\begin{align*}
\text{CH}_3 \\
\text{TATATAAGCA} \\
\text{ATATATTCGT} \\
\text{CH}_3
\end{align*}
\]

\[
\begin{align*}
\text{G:C} \rightarrow \text{A:T}
\end{align*}
\]

\[
\begin{align*}
\text{Cytosine methylation (transcriptional gene silencing)}
\end{align*}
\]

\[
\begin{align*}
\text{Dikaryon DNA synthesis, karyogamy, meiosis}
\end{align*}
\]

\[
\begin{align*}
\text{Meiotic products}
\end{align*}
\]

**Figure 1.5:** RIP in Neurospora. In the dikaryon, the nucleus having the duplicated segment (Chromosomes depicted in blue colour and the duplicated segment as a fluorescent green box) is targeted by the RIP machinery, which induces G:C to A:T mutations in both the copies. The mutated sequence is depicted in pink colour. This is followed by methylation of most of the remaining cytosine residues. The mutations segregate in the progeny which are meiotic products of the cross. The nucleus that does not have the duplication (with chromosomes depicted in maroon colour) is not subjected to such mutations. In the sequences depicted, the blue residues get changed to the red ones after one round of RIP. Adapted and modified from Galagan and Selker, Trends Genet. 2004 Sep;20(9):417-23.
1.5.1 Probable mechanisms of RIP

RIP occurs in a pairwise manner therefore it must involve a mechanism to detect the duplicated sequences during the premeiotic mitoses. Studies done on Arabidopsis and budding yeast have suggested that chromosomal loci with transgenic sequences are more often associated with each other than the normal loci (Fuchs et al., 2002; Pecinka et al., 2005). Both copies of a duplicated sequence are silenced in an irreversible manner in RIP therefore it is suggested that premeiotic pairing is a prerequisite for this mechanism.

The high frequency of RIP mutations suggests that it is an enzymatic process. Three models have been proposed to explain the mechanism. A DNA-cytosine deaminase or a methyl transferase has been suggested that catalyzes the conversion of cytosine to uracil. The uracil DNA-glycosylase fails to repair the mismatch probably because the paired DNA structure does not allow for the detection of the lesion. Consequently, the uracils are substituted by thymines during replication (Fig 1.6 A). Alternatively the uracil intermediate may be avoided by methylation of cytosine followed by deamination to a thymine by the enzyme 5m-cytosine deaminase (Fig 1.6 B). Both these events can be catalysed by a cytosine-DNA methyl transferase (Selker, 1990; Mautino and Rosa, 1998). A third mechanism is by enzymatic methylation of cytosine, to give a reactive intermediate species, 5,6-dihydrocytosine which is extremely energetic and thus exists as a complex with the methyl transferase. The complex is a result of a nucleophilic attack on the C6 position of the cytosine. As a result, a negative charge forms on C5 of cytosine which makes it capable of accepting a methyl group from S-adenosylmethionine (SAM). This would result in formation of 5mC, which would get deaminated to form thymine (Fig. 1.6 C). Alternatively, tautomerization of the intermediate to an imino group and its hydrolysis can yield uracil. Thus the cellular levels of SAM may determine the fate of the cytosines (Mautino and Rosa 1998, Rosa et al., 2004).

Gene silencing by RIP occurs in an RNAi- dependent and RNAi- independent manner.

RNAi targets transcriptional or post-transcriptional silencing of genes by either locus-specific methylation of Lys9H3 or by mRNA degradation (Volpe et al., 2002; Hall et al., 2002; Pal-Bhadra et al., 2004). Lys9H3 methylation of RIPed sequences in N.
Figure 1.6: Models to explain the conversion of G:C to A:T.

A- Hypothesis involving the enzyme DNA-cytosine deaminase which catalyzes the conversion of cytosine to uracil.

B- Hypothesis that involves methylation of cytosine to 5m cytosine followed by deamination to thymine by a 5m-cytosine deaminase.

C- This model involves the formation of a reactive intermediate species, 5,6 dihydrocytosine and its conversion to a 5mC by accepting a methyl group from S-adenosylmethionine (SAM) which gets deaminated to a thymine.
crassa is carried out by DIM-5 (Tamaru and Selker, 2001), which lacks a chromodomain that in association with siRNA is used to recognize sites to be methylated (Bannister et al., 2001). Therefore Lys9H3 methylation by DIM-5 might involve recognition of specific A:T content of the sequence that results from the action of RIP on duplicated sequences. Further, the RIPed sequences are transcribed and are thus targets for post-transcriptional gene-silencing. When the RNAi requiring genes are knocked out, the level of transcripts from the RIP-mutated regions increases. This suggests that the transcripts from RIPed sequences are post-transcriptionally silenced (Chicas et al., 2004, 2005). Therefore maintaining the silenced state of the RIPed sequences by post-transcriptional silencing is carried out in an RNAi-dependent manner although the transcriptional silencing is independent of components required for RNAi.

1.5.2 Measuring RIP

A RIP assay has been developed in our lab, which makes use of the transgenic strain Dp(erg-3/1), which contains a 1.3 kb fragment duplication of the erg-3 gene and was created by transformation (See chapter 2 for details). The erg-3 gene codes for a sterol C-14 reductase. The duplication targets RIP to the endogenous copy of the erg-3 gene and crosses of Dp(erg-3/1) strains with the wild type produce RIP induced erg-3 mutants. Ascospores mutant in erg-3 produce colonies with a distinct morphology on sorbose supplemented medium, which makes them very easy to score. Frequency of erg-3 mutants obtained in the progeny provides a measure of the RIP frequency. This assay has been extensively used in our lab to identify strains that behave as dominant suppressors of RIP (Fig 1.7).

1.5.3 Suppression of RIP

A putative cytosine methyltransferase gene was identified based on its homology to the Ascopolus immersus MascI gene which when mutated causes crosses to be defective for MIP (methylation induced premeiotically). This gene was designated as rid-1 (RIP defective-1; Freitag et al., 2002). Although it shares homology with methyl transferase, its methyl transferase activity has not yet been demonstrated. Crosses homozygous mutant for rid-1 are RIP defective. Using this mutation, Bhat and Kasbekar
Fig 1.7: Phenotype of the *erg-3* mutant ascospore colony on sorbose medium. When a *Dp(erg-3/1)* strain is crossed to a wild type strain, ectopic copy of the *erg-3* gene targets RIP to the endogenous copy and produces RIP-induced mutants among the progeny from this cross. The two types of colonies, *erg-3* mutant and wild-type are marked with arrows and they can be easily distinguished. A count of the number of such mutants vs the total number of progeny provides a measure for the frequency of RIP. Image from lab sources.
(2004) have established that RIP is not responsible for the barren phenotype of crosses heterozygous or homozygous for large chromosome segment duplications.

*Naturally occurring dominant suppressors of RIP.*

Wild isolates have been pivotal in identifying active and also relic transposable elements. A screen done in our lab of 400 different wild isolated strains led to the identification of seven that dominantly suppress RIP. Of these, Adiopodoumé-1 is the only *N. crassa* strain that has active copies of the transposable element Tad. Defective RIP or the insensitivity of the Tad element to the action of RIP could possibly be the cause for the survival of Tad in Adiopodoumé-1. Rapid loss of the Tad elements in sexual crosses (Anderson et al., 2001) and the work by Kinsey and co-workers on Tad and the *am* gene suggested that Tad is susceptible to RIP and also seems to demonstrate that Adiopodoumé-1, is capable of RIP (Kinsey et al., 1994). However results from our lab using the RIP assay suggest that Adiopodoumé-1 is a dominant suppressor of RIP (Noubissi et al., 2000; Noubissi et al., 2001, Bhat et al., 2003), which probably is the reason why Tad has survived in this wild isolate. Subsequently it has also been demonstrated in our lab that the dominant RIP suppressor phenotype can be epigenetically lost among the progeny of crosses involving Adiopodoumé-1 (Tamuli and Kasbekar, unpublished results) and this could possibly explain the discrepant results of Kinsey and co-workers. Epigenetic regulation of the suppressor phenotype is speculated from the fact that the suppressor phenotype can be recovered among progeny of crosses involving parent strains that had initially shown evidence for loss of this phenotype. The dominant RIP suppressor of Adiopodoumé-1 is linked to the mating type and has been mapped to a 34 kb region by my colleague Ranjan Tamuli. Interestingly the 34 kb region includes the *upr-1* gene which encodes the catalytic subunit of translesion DNA Pol zeta. Studies are being done to determine whether the *upr-1* gene is responsible for the dominant RIP suppression in Adiopodoumé-1. A parallel study done in our lab has shown that the translesion polymerases are not required for RIP (Sakai et al., 2002; Tamuli et al. 2006). Of the dominant suppressors of RIP identified in the screen involving the wild-isolated strains, we suspect one, Sugartown to be a naturally occurring duplication because of the barren phenotype associated with the dominant suppressor.
Chromosome segment duplications in Neurospora are barren in crosses and they also exhibit certain phenotypes that are not characteristic to the parental strains from which they are derived.

1.5.4 Large chromosome segment duplications

Chromosomal rearrangements have been useful in studying recombination, position effects, mutations, transvection and meiotic silencing (McClintock, 1938a, 1938b, 1939, 1941; Aramayo and Metzenberg, 1996; Shiu et al., 2001; Shiu and Metzenberg, 2002). They can be generated by recombination events involving multiple copies of transposable elements that might be present in the genome. They may also occur spontaneously due to chromosome breakage and due to exposure to mutagens. Rearrangements can be detected by altered segregation of markers, which already have their linkage group and map position established. They can be detected also based on the ascospore abortion pattern (Perkins, 1962; 1974). Chromosomal rearrangements have been identified in Neurospora and many have been well studied. Insertional or quasi-terminal chromosomal rearrangements can produce duplication progeny in crosses with normal sequence strains. Duplication generating rearrangements can be used to determine the order of genes and also help in studying the behavior of the het genes (Newmeyer and Galeazzi, 1977 and 1978; Perkins et al., 1986). Duplications involving the nucleolar organizing region (NOR) regions are very unstable and breakdown rapidly (Butler, 1989, 1992; Perkins et al., 1995). This mainly happens by somatic recombination and is termed as haploidization of the diploid (Perkins and Barry, 1977; Smith, 1974). Presence of a large chromosome segment duplication can bring about changes in the genome architecture which may influence efficiency of genome defense mechanisms in Neurospora.

Duplications as dominant suppressors of RIP

A study done in the lab showed that large chromosome segment duplications behave as dominant suppressors of RIP in small gene-sized duplication (Bhat and Kasbekar, 2001; Vyas et al., 2006). This might probably be due to titration of the limiting amount of the RIP machinery (Bhat and Kasbekar, 2001; Fehmer et al., 2001;
Vyas et al., 2006). Estimating the size of such large duplications may provide an idea about the minimum size of a large duplication required for dominant RIP-suppression. The genome sequence can be used for mapping the breakpoints of translocations and determining the extent of large duplications. Since standard laboratory strains do not have polymorphism for such analysis, wild-isolated strains can be used as a source of RFLPs (this piece of work is described in Chapter 3 of the thesis).

*Duplication strains used in the study and details of the rearrangements from which they were derived by means of crosses.*

*Dp(AR17):*

The complex translocation $T(AR17)$ involving IR, IIR and IIIR was obtained in a pyr-1 met-1 a strain by UV mutagenesis. Spore development is abnormal in some asci in crosses involving the traslocation. The translocation is homozygous fertile and produces duplication $Dp(AR17)$ progeny in crosses with a normal sequence strain which represent one third of the surviving population. This duplication characteristically segregates RIP-induced dow mutants in crosses with euploid strains because it includes the dow gene. Crosses involving the duplication are stably barren. The ascospores that are produced have peculiar shape and are also oversized (Perkins and Barry, 1977, Perkins, 1997).

*Dp(AR18):*

$T(AR18)$ is an insertional translocation involving IIL and IIIR. The translocation is homozygous fertile. The rearrangement involves the het-6 (Smith and Glass, 1994) gene which controls heterokaryon incompatibility (Mylyk, 1975; Perkins et al., 1993a). It was obtained by UV mutagenesis of the pyr-1 met-1 a strain. It produces slow growing duplication $Dp(AR18)$ progeny in crosses with euploid strains at one third the frequency of the surviving strains. The duplications are stably barren in crosses with non-duplication strains (Perkins and Barry, 1977; Perkins, 1997).

*Dp(IBj5):*

The insertional traslocation $T(IBj5)$ just overlapping the 3' UTR region of the cpc-1 gene involves IVL and IR. It was originally obtained as a cpc-1 mutant allele in a UV
mutagenesis experiment. The mutation was later shown to be lacking in the cpc-1 mRNA and shown to have a translocation breakpoint within the promoter region (Paluh et al., 1990). One third of the viable progeny from the T x N cross are duplication Dp(IBj5) which are phenotypically cpc-1⁺. The duplications are slow growing and extremely barren in crosses with euploid strains (Perkins, 1997).

\textit{Dp(OY329):}  
\textit{T(OY329)} is an insertional translocation of a segment of VIR to IIIR. The translocation is homozygous fertile and was obtained by UV mutagenesis of an \textit{al-2} mutant. One third of the viable progeny from T x N cross are \textit{Dp(OY329)} and show a stably barren phenotype in crosses with non-duplication strains (Perkins, 1997).

\textit{Dp(B362i):}  
The translocation \textit{T(B362i)} involving IVR and I was obtained in the same strain having \textit{arg-10} mutation and the unlinked translocation \textit{T(B362r)} using STA and gamma rays as mutagens. The translocation homozygous cross has reduced fertility. One third of the viable progeny from T x N cross are \textit{Dp(B362i)} duplication progeny. They are stably barren in crosses with non-duplication strains (Perkins and Barry, 1977; Perkins, 1997).

\textit{Dp(S1229):}  
\textit{T(S1229)} is a complex translocation involving IV, VII, I, II and IV. The strain of origin was \textit{pe fl Y8743-21-(13-7) a} and was a result of X-ray mutagenesis (Barratt et al., 1954, Barry and Perkins, 1969). The translocation is homozygous barren. Duplications can be obtained from Tx N cross and they are stably barren in crosses. The aberration cannot be separated from the \textit{pe} mutation.

\textit{RIP-susceptibility of a duplicated sequence}  
Certain regions of the genome are immune to RIP such as, the NOR. Most of the rRNA genes that are present in multiple copies occupy this region and it probably accounts for their ability to escape the action of RIP (Selker et al., 1981). Unlike, other
eukaryotic organisms, the 5S rRNA genes in *N. crassa* are not arranged tandemly and are scattered throughout the genome. Neurospora has probably evolved this characteristic to avoid these sequences from getting RIPed. There are also other factors that may determine the ability of a sequence to be acted upon by RIP. The sequence composition or the flanking region of a duplicated sequence may play a role in rendering a target sequence more susceptible to the action of RIP. Some of these aspects have been probed and are described in Chapter 4 of the thesis. Not only the duplicated sequences but also their flanking regions are targets for RIP-induced gene-silencing. Although RIP seems to be highly regulated there have been instances where RIP was shown to spill over into the flanking regions of a duplicated sequence (Foss *et al.*, 1991; Irelan *et al.*, 1997; Vyas and Kasbekar, 2005). In this thesis, a study has been done to find out the efficiency of spread of RIP and has been described in Chapter 5.

### 1.5.5 RIP in other systems

RIP has been reported in other fungi like *Podospora anserina* (Hamann *et al.*, 2000; Graia *et al.*, 2001), *Magnaporthe grisea* (Ikeda *et al.*, 2002) *Leptosphaerella maculans* (Idnurm and Howlett, 2003), *Microbotryum violaceum* (Hood *et al.*, 2005) and more recently in *Aspergillus oryzae* (Monteil *et al.*, 2006), *Fusarium oxysporium* and *Aspergillus nidulans* (Clutterbuck *et al.*, 2004). Although RIP is active in Podospora and Magnaporthe, it is not very efficient and occurs at a very low frequency. A deletion allele of *ami*, a gene involved in nuclear distribution causes elevation the RIP frequency in *P. anserina* (Bouhouche *et al.*, 2004). In a recent development it has been reported that viral loads in some patients infected with HIV or HBV were considerably reduced because of the activity of APOBEC family enzymes (Muckenfuss *et al.*, 2006). These cytosine deaminases are known to introduce G to A hypermutations leading to inactivation and silencing of retroviruses. Thus, RIP like genome defense mechanisms may exist in mammalian systems as well.

### 1.5.6 Evolutionary consequences of RIP

Genomic duplications serve as a means for gene evolution and presence of RIP has deprived Neurospora of this privilege. If at all gene evolution has occurred in
Neurospora, it might be by means that do not involve duplications, like spontaneous mutations, random deletions, rearrangements etc. RIP may be responsible for the single copy status of the Neurospora genes that are represented as small families of closely related or identical genes in other organisms. For example, Neurospora has single copy of histone genes H2A, H2B and H3 and they contain intronic sequences. This is in contrast to the intronless multiple copies present in other eukaryotes. (Galagan et al., 2003; Borkovich et al., 2004; Galagan and Selker, 2004, Hynes, 2004) This could be a consequence of natural selection of those individuals where the intronic inserts fragmented the homology stretches to an extent such that they were below the threshold level required for detection by the RIP machinery (Hays et al., 2002). Neurospora has also developed other genome defense mechanisms that occur at a later stage in its life cycle to check the proliferation of transposable elements that might have escaped the effects of RIP or prevent transfer of chromosomal rearrangements.

1.6 Meiotic silencing

A presumed RNAi -based genome defense process is active during meiosis in Neurospora. During meiosis, each allele is able to sense the presence of its partner on the homologous chromosome, failing which, silencing of all copies of that unpaired DNA (regardless of whether the other copies are paired or unpaired) is triggered by a silencing mechanism called meiotic silencing (Aramayo and Metzenberg, 1996; Shiu et al., 2001). Studies with the *Asm-1* loss of function mutants established that these mutants fail to develop (Aramayo and Metzenberg, 1996). Further all spores fail to develop in an *Asm-1* deletion x wild type cross indicating the ascus dominance of the *Asm-1* deletion allele. Later it was found that pairing of the *Asm-1* gene was an absolute essential for normal development (Aramayo and Metzenberg, 1996; Aramayo et al., 1996). Shiu et al (2001), solved the enigma as to how a deletion allele of a gene can be dominant with the discovery of meiotic silencing (Fig. 1.8). Like quelling which occurs in the vegetative stage of Neurospora, meiotic silencing requires unpaired transcribed coding sequences. The unpaired copies cause siRNAs to form which then bring about silencing by mRNA degradation of the duplicated genes. Genes required for meiotic silencing are *sad-1, sad-2, sms-2* and *sms-3*, which code for an RdRP, a RISC assembly protein, an Argonaut like
Figure 1.8: Proposed model for meiotic silencing of unpaired DNA (MSUD). During meiosis, a DNA fragment that unpaired DNA triggers the second RNA silencing pathway in *N. crassa*, called MSUD. Mechanism of silencing in MSUD is supposed to be quite similar to that in quelling except that MSUD uses a different set of silencing protein components (paralogs) from those in the quelling pathway. Figures taken from Nakayashiki et al., 579 (2005) 5950-5957
protein and a Rec-Q helicase respectively. Since the RNAi signal can transmit through the cytoplasm, it spreads throughout the syncitium of the ascus thus silencing the gene in all nuclei. The silencing induced by unpaired DNA does not affect the neighboring gene (Kutil et al., 2003). Meiotic silencing has also been reported in mammals where unsynapsed chromosomal material is silenced (Barrends et al., 2005). Genes required for meiotic silencing have been identified in genetic screen and also by candidate gene approach. Mutations that affects meiotic silencing can change the phenotypes of ascus dominant markers like Asm-1, Round spore, Peak, Near round and Banana. Mutations in the genes mentioned below can suppress meiotic silencing.

1.6.1 Suppression of meiotic silencing

Sad-1

sad-1 (suppressor of ascus dominance), codes for an RdRP. A semi-dominant mutation in the sad-1 gene causes suppression of meiotic silencing. This is because the deletion alleles or the severely RIPed alleles of Sad-1 mutants fail to pair with the wild-type copy and this triggers the silencing of the wild type copy. Generally, duplication-heterozygous crosses in Neurospora have a barren phenotype (Perkins and Barry, 1977). Careful observations of nine different duplication-heterozygous crosses showed that perithecial development is initiated but sexual development is arrested before meiosis (Raju and Perkins, 1978). It was earlier proposed to be due to RIP which may cause heterochromatinization and block DNA replication or the RIP induced changes in the duplicated sequence may give rise to a product which may poison the cell and lead to barrenness (Selker, 1990). The barrenness is caused by meiotic silencing of the genes that are included in the duplication which remain unpaired during meiosis (Shiu et al., 2001). Some of these might be required for ascus development or ascospore maturation thereby rendering duplication-heterozygous cross barren.

Sad-2

sad-2 is the most recent addition to the list of genes required for meiotic silencing; it is required for the perinuclear localization of SAD-1 (Fig 1.9). Like the other meiotic silencing requiring genes, null alleles of sad-2 are semi-dominant and a homozygous Sad-2 cross is non-productive (Shiu et al, 2006). Chui et al (2004) have shown that
Figure 1.9: Suppression of ascus-dominant phenotypes by semi-dominant mutations for meiotic silencing. Sad-1 can suppress ascus dominant phenotypes. In panel A, a round spore strain, R produces only round spores in a cross with a wild type strain because it is an ascus dominant mutation. Panel B shows a cross of the round spore strain, R with a Sad-1 mutant strain Sad-1 suppresses ascus dominance of R and therefore produce mostly spindle shaped spores. Image taken from Shiu et al., (2001).
siRNA localize to the perinuclear region in mammalian cells. Shiu et al (2006) also observed that the SAD-1 and SAD-2 proteins are localized to the perinuclear region. Their work suggests that the SAD-2 protein is localized first to the perinuclear region and then it recruits the SAD-1 protein. It is also suggested that immediate conversion of aberrant RNA into dsRNA may be required for more efficient silencing. *sms-2* is a paralog of the *qde2* encoded Argonaut protein required for quelling. Thus it is part of the meiotic silencing machinery and a deletion allele or a severely defective allele of *Sms-2* suppresses meiotic silencing in a semi-dominant way. *sms-3* codes for a Rec-Q helicase required for meiotic silencing and was identified by scanning the genome sequence.

### 1.6.2 Are large duplications recessive suppressors of meiotic silencing?

Large chromosome segment duplications not only influence the action of RIP but initial experiments suggested that they might also cause recessive suppression of meiotic silencing. Barrenness in the duplication-heterozygous crosses can be suppressed by the semi-dominant suppressors of meiotic silencing. Crosses that are homozygous for large chromosome segment duplications are expected to be fertile since there are no unpaired alleles during meiosis. From the findings of Shiu et al (2001) and studies done in our lab (Bhat and Kasbekar, 2004; Vyas et al., 2006) these crosses were found to be barren. In this thesis, an extensive study has been done on crosses homozygous for large chromosome segment duplications. Interestingly these crosses show a very unusual phenotype. Other interesting observations and speculations for these are discussed in chapter 6. The concept of homology search and pairing between chromosomes has significance in this regard.

### 1.6.3 Homolog pairing and meiotic silencing

Interplay of homology recognizing mechanisms may vary greatly in different organisms. One mechanism may be predominant in a particular organism and missing in another (Lui et al., 2006). The proteinaceous synaptonemal complex brings about stable juxtaposition of homologous chromosomes in female *D. melanogaster* and *C. elegans* whereas recombination stabilizes the homologs in *Sordaria*, *Zea mays*, *Mus musculus* and *Saccharomyces cerevisiae* (McKim et al., 2002; Mahadevaiah et al., 2001; Peoples et al.,
In S. pombe, telomere pairing plays an important role in homolog pairing (Ding et al., 2004). Recognition of DNA/DNA homology before and during meiosis also plays an important role in detecting DNA segments that are present in inappropriate number of copies.

**Trans-sensing**

Trans-sensing is a very important aspect of homolog pairing and in Neurospora, mei-3, a Rad-51 homolog, might fulfill this function (Bean et al., 2004; Bowering et al., 2006). In Neurospora it may be during the early pairing that the unpaired copies of any duplicated sequence or transgenes are detected and meiotically silenced. The trans-sensing of homolgs is affected by methylation as shown by Pratt et al., (2004). They demonstrated that meiotic silencing and trans sensing mechanisms can be genetically dissected using very sensitive assays. RIPed alleles of ascus dominant Round spore (R) in the background of a dim-2 (methylation disrupted) mutant showed reduced silencing (fewer number of round spores) in contrast to the same alleles in a dim-2\(^{+}\) background. Thus the methylation status of the alleles determined their trans-sensing efficiency (Pratt et al, 2004). Their finding suggests two models for trans sensing, DNA identity where a methylated base would be recognized as a non-match and chromatin identity in which, a euchromatic region paired against a heterochromatic region would trigger meiotic silencing. Meiotic silencing of unpaired sequences is more efficient when the length of the unpaired fragment is more and there is increased homology between the interacting sequences (Lee et al., 2004). These possibilities have been implicated in some of the results that I have obtained for crosses that are homozygous for large chromosome segment duplications, heterozygous for Sad-1 and homozygous for the rid-1 mutation in chapter 6 of the thesis.

### 1.6.4 Meiotic silencing in other systems

Recently it has been demonstrated that transcriptional silencing of sex chromosome during male meiosis is induced due to asynapsis of either whole or part of the chromosome (Turner et al., 2005; 2006). Female meiosis also involves this phenomenon. Meiotic silencing of unsynapsed chromosomes (MSUC) has been
demonstrated in male and female mouse where unsynapsed parts of chromosomes induce their meiotic silencing (Barrends et al., 2005). MSUC is also accompanied by a post-meiotic repression of the silenced chromosome. Meiosis may involve many control and regulatory mechanisms that may come into play in case of chromosomal rearrangements and thus the checkpoints may lead to silencing of the genes by incorporating modified histones or ubiquitination of the histones (Barrends et al., 2005). In C. elegans, unpaired DNA leads to accumulation of high levels of histone H3 lysine 9 dimethylation (H3K9me2) mediated by ego-1, an RNA-dependent RNA polymerase (Smardon et al., 2000; Maine et al., 2005). This modification is generally associated with formation of facultative heterochromatin and transcriptional silencing (Bean et al., 2004, Kelly et al., 2002). Existence of these mechanisms in organisms belonging to different levels in the phylogeny suggests that these mechanisms are quite conserved although the mode of action is not the same in all organisms exhibiting it.

1.6.5 Significance of meiotic silencing

With the discovery of MSUC, MSUD and other related phenomenon in various organisms, it is increasingly evident that a general meiotic silencing pathway exists in most organisms, which to a large extent prevents the transfer of rearrangements to the next generation. Meiotic silencing in oocytes may also be a mechanism of scanning the meiotic cells for their relative fitness. Requirement of RNA silencing machinery in meiotic silencing suggests that it may have a yet undiscovered role to play in meiotic regulation of genes by production of micro RNAs etc. (Ambros, 2001; Lagos-Quintana et al, 2001). Like the siRNA, another class of small RNAs, miRNA cause transcriptional suppression of the target mRNA with which it has imperfect or perfect complimentarity (Chen and Meister, 2005). With regard to implication of meiotic silencing and miRNA in development, it is speculated that both mechanisms may have evolved from an ancient silencing machinery (reviewed by Nakayashiki, 2005).

Meiotic silencing can play a subtle role in evolution and speciation. Lee et al. (2004) have suggested that two closely related genomes that are coevolving may accumulate silent rearrangements (rearrangements that do not affect meiosis, ascospore development or maturation) and still maintain their interbreeding ability. Any
rearrangement involving a gene critical for any of these functions might result in their reproductive isolation. Genomic stability is greatly enhanced by such surveillance. Micro heterogeneity between isolated populations may be tolerated by meiotic silencing but further accumulation of such difference might trigger meiotic silencing leading to intrapopulation crosses being rendered barren (reviewed by Catalanotto, 2006). In fungi like Neurospora, it has been speculated that meiotic silencing of the polymorphic alleles is responsible for the sexual block. Such blocks can be overcome by making the cross defective for meiotic silencing (Shiu et al., 2001). Interspecies crosses heterozygous for Sad-I produce progeny early enough and in significantly greater number as compared to interspecies crosses between the wild type strains.

1.7 Gene evolution in Neurospora

In most organisms, a major way of evolving new genes is through duplication. Neurospora has very little such privilege if not none because of the genome defense mechanisms that are operational at various stages in its life cycle (Galagan et al., 2003; Galagan and Selker, 2004). New genes have evolved very rarely leading to a prolonged evolutionarily eventless phase. This could happen if RIP is suppressed in certain backgrounds like crosses that harbor large chromosome segment duplications and some wild-isolated strains which behave as dominant suppressors of RIP (Noubissi et al., 2000; Noubissi et al., 2001, Fehmer et al., 2002, Bhat et al., 2003). Any small duplicated sequences in such backgrounds can escape the effects of RIP and potentially evolve into new genes (Bhat and Kasbekar, 2001; Vyas et al., 2006). Some aspects of the genome defense mechanisms in Neurospora have been addressed and discussed in further detail in the following chapters in this thesis.